RayBio® C-Series
Human Cytokine Antibody Array C11

For the semi-quantitative detection of 37 human cytokines in serum, plasma, cell culture media and other liquid samples types

Patent Pending Technology

User Manual
(Revised October 28, 2014)

Cat# AAH-CYT-11-2 (2 Sample Kit)
Cat# AAH-CYT-11-4 (4 Sample Kit)
Cat# AAH-CYT-11-8 (8 Sample Kit)

Please read manual carefully
before starting experiment

RayBiotech, Inc.
the protein array pioneer company

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C-Series Antibody Arrays

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I. INTRODUCTION

New techniques such as cDNA microarrays have enabled us to analyze global gene expression[1-3]. However, almost all cell functions are executed by proteins, which cannot be studied simply through DNA and RNA techniques. Experimental analysis clearly shows disparity can exist between the relative expression levels of mRNA and their corresponding proteins[4]. Therefore, analysis of the proteomic profile is critical.

The conventional approach to analyzing multiple protein expression levels has been to use 2-D SDS-PAGE coupled with mass spectrometry [5,6]. However, these methods are slow, expensive, labor-intensive and require specialized equipment[7]. Thus, effective study of multiple protein expression levels can be complicated, costly and time-consuming. Moreover, these traditional methods of proteomics are not sensitive enough to detect most cytokines (typically at pg/ml concentrations).

Cytokines, broadly defined as secreted cell–cell signaling proteins distinct from classic hormones or neurotransmitters, play important roles in inflammation, innate immunity, apoptosis, angiogenesis, cell growth and differentiation[7]. They are involved in most disease processes, including cancer, obesity and inflammatory and cardiac diseases.

Simultaneous detection of multiple cytokines undoubtedly provides a powerful tool to study cell signaling pathways. Regulation of cellular processes by cytokines is a complex, dynamic process, often involving multiple proteins. Positive and negative feedback loops, pleiotrophic effects and redundant functions, spatial and temporal expression of or synergistic interactions between multiple cytokines, even regulation via release of soluble forms of membrane-bound receptors, all are common mechanisms modulating the effects of cytokine signaling[8-14]. As such, unraveling the role of individual cytokines in physiologic or pathologic processes generally requires consideration and detection of multiple cytokines rather than of a single cytokine.

RayBio® C-Series Antibody Arrays have several advantages over single-target ELISA kits:

1. More Data, Same or Less Sample: Antibody arrays provide high-content screening using about the same sample volume as traditional ELISA.
2. Global View of Cytokine Expression: Antibody array screening improves the chances for discovering key factors, disease mechanisms, or biomarkers related to cytokine signaling.
3. Similar (sometimes better) Sensitivity: As little as 4 pg/ml of MCP-1 can be detected using the C-Series array format. In contrast, our similar MCP-1 ELISA assay has a sensitivity of 40 pg/ml of MCP-1.
4. Wider Detection Range: ELISA typically detects a concentration range of 100 to 1000-fold, while RayBiotech arrays can detect concentration ranges up to 10,000-fold.
5. Better Precision: As determined by densitometry, the inter-array Coefficient of Variation (CV) of spot signal intensities is 5-10%, comparing favorably with ELISA testing (CV = 10-15%).

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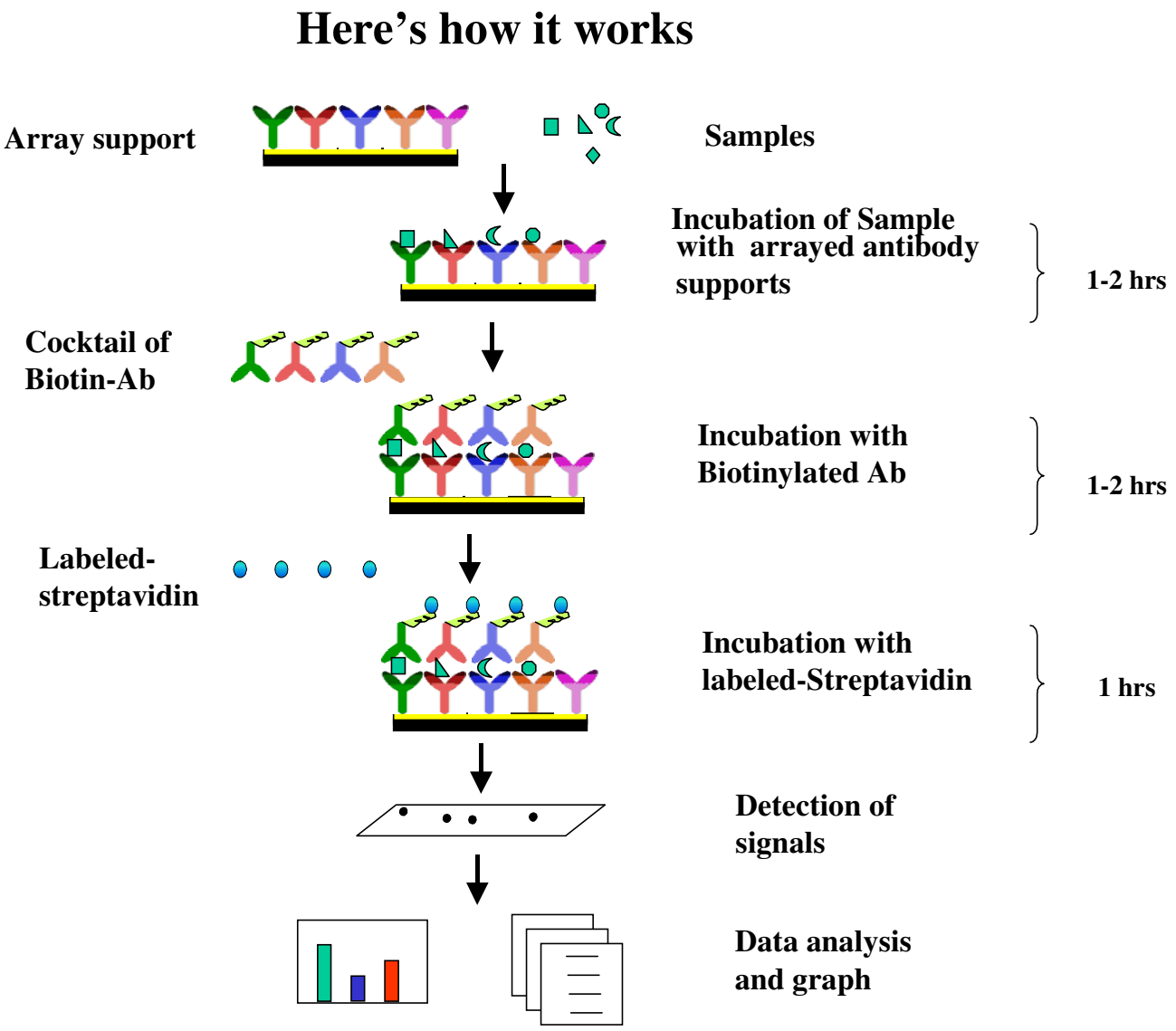
II. HOW IT WORKS



III. COMPONENTS AND STORAGE

Store kit at ≤ -20 °C immediately upon arrival. Kit must used within the 6 month expiration date.

ITEM	COMPONENT	AAC-CYT-1-2	AAC-CYT-1-4	AAC-CYT-1-8	STORAGE TEMPERATURE AFTER THAWING**
1	Antibody Arrays	2 membranes	4 membranes	8 membranes	≤-20 °C
2	Blocking Buffer	1 vial (25 ml)		2 vials (25 ml/ea)	
3	Biotinylated Antibody Cocktail	2 vials		4 vials	2-8 °C (for up to 3 days after dilution)
4	1,000X HRP-Streptavidin Concentrate	1 vial (50 µl)			2-8 °C
5	20X Wash Buffer I Concentrate	1 vial (10 ml)		1 vial (20 ml)	
6	20X Wash Buffer II Concentrate	1 vial (10 ml)		1 vial (20 ml)	
7	2X Cell Lysis Buffer Concentrate	1 vial (10 ml)		1 vial (16 ml)	
8	Detection Buffer C	1 vial (1.5 ml)		1 vial (2.5 ml)	
9	Detection Buffer D	1 vial (1.5 ml)		1 vial (2.5 ml)	
10	8-Well Incubation Tray w/ Lid	1 tray			Room Temperature
Other Kit Components: Plastic Sheets, Array Map Template, User Manual					

*Each package contains 2 or 4 membranes

**For up to 3 months (unless stated otherwise) or until expiration date

IV. ADDITIONAL MATERIALS REQUIRED

- Pipettors, pipet tips and other common lab consumables
- Orbital shaker or oscillating rocker
- Tissue paper, blotting paper or chromatography paper
- Adhesive tape or plastic wrap
- Distilled or de-ionized water
- A chemiluminescent blot documentation system:
  - CCD Camera
  - X-Ray Film and a suitable film processor
  - Gel documentation system
  - Or another chemiluminescent detection system capable of imaging a western blot

V. SAMPLE TIPS AND GENERAL CONSIDERATIONS

A. Sample Collection, Preparation, and Storage

NOTE: *Optimal methods will need to be determined by each experimenter empirically based on researched literature and knowledge of the samples.*

- If not using fresh samples, freeze samples as soon as possible after collection.
- Avoid multiple freeze-thaw cycles. If possible, sub-aliquot samples prior to initial storage.
- Serum-free or low serum containing media (0.2% FBS/FCS) is recommended. If serum containing media is required, testing an uncultured media sample as a negative control is ideal as many types of sera contain cytokines, growth factors and other proteins.
- It is strongly recommended to add a protease inhibitor cocktail to cell and tissue lysate samples.
- Avoid using EDTA as an anti-coagulant for collecting plasma if testing MMPs or other metal-binding proteins.
- Avoid using hemolyzed serum or plasma as this may interfere with protein detection and/or cause a higher than normal background response.
- Avoid sonication of 1 ml or less as this can quickly heat and denature proteins
- Most samples will not need to be concentrated. If concentration is required, a spin column concentrator with a chilled centrifuge is recommended.
- Always centrifuge the samples hard after thawing (~10,000 RPM for 2-5 minutes) in order to remove any particulates that could interfere with detection.
- ***General tips for preparing serum, plasma, cell culture media, urine, and lysate samples can be viewed on the online Resources page of the website.***

B. Sample Types and Recommended Dilutions/Amounts

NOTE: *Optimal sample dilutions and amounts will need to be determined by each experimenter empirically but the below recommendations may be used as a starting point. Blocking Buffer (ITEM 2) should be used to dilute samples if necessary. Normalize samples by loading equal amounts or equal dilutions.*

- **Cell Cultured Media**: Neat (no dilution needed)
- **Serum & Plasma**: 2-fold to 10-fold dilution
- **Other Body Fluids and Liquids**: Neat or 2-fold to 5-fold dilution
- **Cell and Tissue Lysates**: load 50 to 500 µg of total protein (after a 5-fold to 10-fold dilution to minimize the effect of any detergent(s). Therefore the original lysate concentration should be 1 to 5 mg/ml.

C. Handling Membranes

- The antibody printed side of each membrane is marked by a dash (-) or number (#) in the upper left corner.
- Do not allow membranes to dry out during the experiment or they may become fragile and break OR high and/or uneven background may occur.
- Grasp membranes by the corners or edges only using forceps. DO NOT touch printed antibody spots.

D. Incubations and Washes

- Perform **ALL** incubation and wash steps under gentle rotation or rocking motion (~0.5 to 1 cycle/sec) using an orbital shaker or oscillating rocker to ensure complete and even reagent/sample coverage. Rocking/rotating too vigorously may cause foaming or bubbles to appear on the membrane surface which should be avoided.
- All washes and incubations should be performed in the Incubation Tray (ITEM 10) provided in the kit.
- Cover the Incubation Tray with the lid provided during all incubation steps to avoid evaporation and outside debris contamination.
- Ensure the membranes are completely covered with sufficient sample or reagent volume during each incubation.
- Avoid forceful pipetting directly onto the membrane; instead, gently pipette samples and reagents into a corner of each well.
- Aspirate samples and reagents completely after each step by suctioning off excess liquid with a pipette. Tilting the tray so the liquid moves to a corner and then pipetting is an effective method.
- Optional overnight incubations may be performed for the following steps to increase overall spot signal intensities:
  - Sample Incubation
  - Biotinylated Antibody Cocktail Incubation
  - HRP-Streptavidin Incubation

NOTE: *Overnight incubations should be performed at 4 °C (also with gentle rocking/shaking). Be aware that longer incubations can also increase the background response so complete liquid removal and washing is critical.*

VI. CHEMILUMINESCENCE DETECTION TIPS

- Beginning with adding the detection buffers and ending with exposing the membranes should take no more than 10-15 minutes as the chemiluminescent signals may start to fade at this point.
- Trying multiple exposure times is recommended to obtain optimum results.
- A few seconds to a few minutes is the recommended exposure time range, with 30 seconds to 1 minute being suitable for most samples.

Don’t have time or the equipment to image your membranes? Let the experts at RayBiotech image and analyze your membranes. Contact us for pricing details.

VII. COMPONENT PREPARATION

NOTE: *Thaw all reagents to room temperature immediately before use. If wash buffers contain visible crystals, warm to room temperature and mix gently until dissolved.*

NOTE: *The Biotinylated Antibody Cocktail (ITEM 3) and the HRP-Streptavidin Concentrate (ITEM 4) vials should be briefly centrifuged (~1000 g) before opening to ensure maximum recovery and mixed well as precipitates may form during storage.*

ITEM	COMPONENT	PREPARATION	EXAMPLE
1	Antibody Arrays	No Preparation	N/A
2	Blocking Buffer		
3	Biotinylated Antibody Cocktail*	Pipette 2 ml of Blocking Buffer into each vial. Mix gently with a pipette.	N/A
4	1,000X HRP-Streptavidin Concentrate	Dilute 1,000-fold with Blocking Buffer. Mix gently with a pipette.	10 µl of 1,000X concentrate + 9990 µl of Blocking Buffer = 10 ml of 1X working solution
5	20X Wash Buffer I Concentrate	Dilute **each** 20-fold with distilled or deionized water.	10 ml of 20X concentrate + 190 ml of water = 200 ml of 1X working solution
6	20X Wash Buffer II Concentrate		
7	2X Cell Lysis Buffer Concentrate**	Dilute 2-fold with distilled or deionized water.	10 ml of 2X concentrate + 10 ml of water = 20 ml of 1X working solution
8	Detection Buffer C	No Preparation	N/A
9	Detection Buffer D		
10	8-Well Incubation Tray w/ Lid		

*1 vial is enough to test 2 membranes

**Only for use for preparing cell or tissue lysates. General tips for preparing lysates and other common sample types can be found on the online Resources Page

VIII. PROTOCOL

NOTE: *Prepare all reagents and samples immediately prior to use. See Sections V and VII.* ***ALL*** *incubations and washes must be performed under gentle rotation/rocking (~0.5-1 cycle/sec)*

1) Remove the kit from storage and allow the components to equilibrate to room temperature (RT).

2) Carefully remove the Antibody Arrays (ITEM 1) from the plastic packaging and place each membrane (printed side up) into a well of the Incubation Tray (ITEM 10). One membrane per well.

NOTE: *The antibody printed side is marked by a dash (-) or number (#) in the upper left corner.*

A. Blocking

3) Pipette 2 ml of Blocking Buffer (ITEM 2) into each well and incubate for 30 minutes at RT.

4) Aspirate blocking buffer from each well with a pipette.

B. Sample Incubation

5) Pipette 1 ml of diluted or undiluted sample into each well and incubate for 1.5 to 5 hours at RT OR overnight at 4 °C.

NOTE: *Longer incubations can help maximize the spot signal intensities. However, doing so can also increase the background response so complete liquid removal and washing is critical.*

6) Aspirate samples from each well with a pipette.

C. First Wash

NOTE: *The 20X Wash Buffer Concentrates I and II (ITEM 5 and 6) must be diluted 20-fold before use. See Section VII for details.*

7) Wash Buffer I Wash: Pipette 2 ml of **1X** Wash Buffer I into each well and incubate for 5 minutes at RT. Repeat this 2 more times for a total of 3 washes using fresh buffer and aspirating out the buffer completely each time.

8) Wash Buffer II Wash: Pipette 2 ml of **1X** Wash Buffer II into each well and incubate for 5 minutes at RT. Repeat this 1 more time for a total of 2 washes using fresh buffer and aspirating out the buffer completely each time.

D. Biotinylated Antibody Cocktail Incubation

NOTE: *The Biotinylated Antibody Cocktail (ITEM 3) must be prepared before use. See Section VII for details.*

9) Pipette 1 ml of the **prepared** Biotinylated Antibody Cocktail into each well and incubate for 1.5 to 2 hours at RT OR overnight at 4°C.

10) Aspirate biotinylated antibody cocktail from each well.

E. Second Wash

11) Wash membranes as directed in Steps 7 and 8.

F. HRP-Streptavidin Incubation

NOTE: *The 1,000X HRP-Streptavidin Concentrate (ITEM 4) must be diluted before use. See Section VII for details.*

12) Pipette 2 ml of **1X** HRP-Streptavidin into each well and incubate for 2 hours at RT OR overnight at 4°C.

13) Aspirate HRP-Streptavidin from each well.

G. Third Wash

14) Wash membranes as directed in Steps 7 and 8.

H. Chemiluminescence Detection

NOTE: *Do not allow membranes to dry out during detection.*

15) Transfer the membranes, printed side up, onto a sheet of chromatography paper, tissue paper, or blotting paper lying on a flat surface (such as a benchtop).

16) Remove any excess wash buffer by blotting the membrane edges with another piece of paper.

17) Transfer and place the membranes, printed side up, onto a plastic sheet (provided) lying on a flat surface.

NOTE: *Multiple membranes can be placed next to each other and fit onto a single plastic sheet. Use additional plastics sheets if necessary.*

18) Into a single clean tube, pipette equal volumes (1:1) of Detection Buffer C (ITEM 8) and Detection Buffer D (ITEM 9). Mix well with a pipette.

EXAMPLE*: 250 µl of Detection Buffer C + 250 µl of Detection Buffer D = 500 µl (enough for 1 membrane)*

19) Gently pipette 500 µl of the Detection Buffer mixture onto each membrane and incubate for 2 minutes at RT (DO NOT ROCK OR SHAKE). Immediately afterwards, proceed to Step 20.

NOTE: *Exposure should ideally start within 5 minutes after finishing Step 19 and completed within 10-15 minutes as chemiluminescence signals will fade over time. If necessary, the signals can usually be restored by repeating washing, HRP-Streptavidin and Detection Buffers incubations (Steps 11-19)*

20) Place another plastic sheet on top of the membranes by starting at one end and gently "rolling" the flexible plastic sheet across the surface to the opposite end to smooth out any air bubbles. The membranes should now be "sandwiched" between two plastic sheets.

NOTE: *Avoid "sliding" the top plastic sheet along the membranes' printed surface. <u>If using X-ray film, do not use a top plastic sheet so that the membranes can be directly exposed to the film</u>.*

21) Transfer the sandwiched membranes to the chemiluminescence imaging system such as a CCD camera (recommended) and expose.

NOTE: *Optimal exposure times will vary so performing multiple exposure times is strongly recommended. See Section VI for additional details.*

I. <u>Storage</u>

22) To store, without direct pressure, gently sandwich the membranes between 2 plastic sheets (if not already), tape the sheets together or use plastic wrap to secure them, and store at ≤ -20 ^{o}C for future reference.

IX. TYPICAL RESULTS

Typical results obtained with RayBio® C-Series Antibody Arrays

The preceding figures present typical images obtained with RayBio® C-Series Antibody Arrays. These membranes were probed with conditioned media from two different cell lines. Membranes were exposed with Kodak X-Omat® film at room temperature for 1 minute.

Note the strong signals of the Positive Control spots in the upper left and lower right corners. (See below for further details on the control spots.)

The signal intensity for each antigen-specific antibody spot is proportional to the relative concentration of the antigen in that sample. Comparison of signal intensities for individual antigen-specific antibody spots between and among array images can be used to determine relative differences in expression levels of each analyte sample-to-sample or group-to-group.

X. INTERPRETING THE RESULTS

A. Control Spots

Positive Control Spots (POS) – controlled amount of biotinylated antibody printed onto the array. Used for normalization and to orientate the arrays.

Negative Control Spots (NEG) – buffer printed (no antibodies) used to measure the baseline responses. Used for determining the level of non-specific binding of the samples.

Blank Spots (BLANK) – nothing is printed here. Used to measure the background response.

B. Data Extraction

Visual comparison of array images may be sufficient to see differences in relative protein expression. However, most researchers will want to perform numerical comparisons of the signal intensities (or more precisely, signal *densities*), using 2-D densitometry. Gel/Blot documentation systems and other chemiluminescent or phosphorescent detection systems are usually sold as a package with compatible densitometry software.

Any densitometry software should be sufficient to obtain spot signal densities from your scanned images. One such software program, ImageJ, is available for free from the NIH website along with an array plug-in.

We suggest using the following guidelines when extracting densitometry data from our array images:

- For each array membrane, identify a single exposure that the exhibits a high signal to noise ratio (strong spot signals and low background response). Strong Positive Control Spot signals but not too strong that that they are "bleeding" into one another is ideal. The exposure time does not need to be identical for each array, but Positive Control signals on each array image should have similar intensities.
- Measure the density of each spot using a circle that is roughly the size of one of the largest spots. Be sure to use the same extraction circle dimensions (area, size, and shape) for measuring the signal densities on every array for which you wish to compare the results.
- For each spot, use the summed signal density across the entire circle (ie, total signal density per unit area)

C. Data Analysis

NOTE: *RayBiotech offers Microsoft® Excel-based Analysis Software Tools for each array kit for automatic analysis. Please visit the website at www.raybiotech.com or contact us for ordering information.*

Once the raw numerical densitometry data is extracted, the background must be subtracted and the data normalized to the Positive Control signals to analyze.

Background Subtraction: Select values which you believe best represent the background. If the background is fairly even throughout the membrane, the Negative Control Spots (NEG) and/or Blank Spots (BLANK) should be similar and are accurate for this purpose.

Positive Control Normalization: The amount of biotinylated antibody printed for each Positive Control Spot is consistent from array to array. As such, the intensity of these Positive Control signals can be used to normalize signal responses for comparison of results across multiple arrays, much like housekeeping genes and proteins are used to normalize results of PCR gels and Western Blots, respectively.

To normalize array data, one array is defined as "Reference Array" to which the other arrays are normalized to. The choice of the Reference Array is arbitrary.

NOTE: *The RayBio® Analysis Software Tools always designate Array 1/Sample 1 as the Reference Array.*

Next, the simple algorithm below can be used to calculate and determine the signal fold expression between like analytes.

X(Ny) = X(y) * P1/P(y)

Where:
P1 = mean signal density of Positive Control spots on reference array
P(y) = mean signal density of Positive Control spots on Array "y"
X(y) = mean signal density for spot "X" on Array for sample "y"
X(Ny)= normalized signal intensity for spot "X" on Array "y"

For example:

Let's determine the relative expression for IL-6 on two different arrays (Arrays 1 and 2). Let's assume that the duplicate signals for the IL-6 spots on each array are identical (or that the signal intensity used in the following calculation is the mean of the two duplicates spots). Also assume the following:

P1 = 2500
P2 = 2700
IL-6 (1) = 300
IL-6 (2) = 455

Then IL-6(N2) = 455 *2500/2700 = 421.30

The fold increase of IL-6(N2) vs IL-6(1) = 421.3/300 = 1.40-fold increase or a 40% increase in the signal intensity of IL-6 in Array 2 vs. Array 1.

XI. ARRAY MAP

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	2B4/ CD244	ADAM9	APRIL	BMP-2	BMP-9	Cathepsin L	CD200	CD97
2	POS	POS	NEG	NEG	2B4/ CD244	ADAM9	APRIL	BMP-2	BMP-9	Cathepsin L	CD200	CD97
3	Chemerin	C5a	DcR3/ TNFRSF6B	FABP2	FAP	FGF-19	Galectin -3	HGF R/ c-MET	IFN-a/b R2	IGF-II R	IL-1 R6/ IL-1 Rrp2	IL-24
4	Chemerin	C5a	DcR3/ TNFRSF6B	FABP2	FAP	FGF-19	Galectin -3	HGF R/ c-MET	IFN-a/b R2	IGF-II R	IL-1 R6/ IL-1 Rrp2	IL-24
5	IL-33	Kallikrein 14	Legumain	LOX-1/ OLR1	MBL	Neprilysin/ CD10	Notch-1	NOV	Osteoactivin/ GPNMB	PD-1	PGRP-S	Serpin A4
6	IL-33	Kallikrein 14	Legumain	LOX-1/ OLR1	MBL	Neprilysin/ CD10	Notch-1	NOV	Osteoactivin/ GPNMB	PD-1	PGRP-S	Serpin A4
7	sFRP-3	Thrombo-modulin	TLR2	Trans-ferrin	WIF-1	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	POS
8	sFRP-3	Thrombo-modulin	TLR2	Trans-ferrin	WIF-1	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	POS

POS = Positive Control Spot
NEG = Negative Control Spot
BLANK = no spot

NOTE: *Protein alternative names, accession numbers, and official symbols can be accessed on www.raybiotech.com via the Resources Page.*

XII. TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	RECOMMENDATION
No signals (not even the positive controls spots)	Chemiluminescent imager is not working properly	Contact image manufacturer
	Too Short Exposure	Expose the membranes longer
	Degradation of components due to improper storage	Store entire kit at ≤ - 20°C. Do not use kit after expiration date. See storage guidelines.
	Improper preparation or dilution of the HRP-Streptavidin	Centrifuge vial briefly before use, mix well, and do not dilute more than 1000-fold
	Waiting too long before exposing	The entire detection process should be completed in 10-15 minutes
Positive controls spots signals visible but no other spots	Low sample protein levels	Decrease sample dilution, concentrate samples, or load more protein initially
	Skipped Sample Incubation Step	Samples must be loaded after the blocking step
	Too Short of Incubations	Ensure the incubations are performed for the appropriate time or try the optional overnight incubation(s)
Uneven signals and/or background	Bubbles present on or below membrane	Don't rock/rotate the tray too vigorously or pipette the sample or reagent with excessive force
	Insufficient sample or reagent volume	Load enough sample and reagent to completely cover the membrane
	Insufficient mixing of reagents	Gently mix all reagents before loading onto the membrane, especially the HRP-Streptavidin and Biotin Antibody Cocktail
	Rocking/Rotating on an uneven surface while incubating	Rock/rotate on a flat surface or the sample or reagent can "pool" to one side
High background signals or all spots visible	Too much HRP-Streptavidin or Biotinylated Antibody Cocktail	Prepare these signal enhancing components precisely as instructed
	Membranes dried out	Do not let the membranes dry out during the experiment. Cover the incubation tray with the lid to minimize evaporation
	Too High of Sample Protein Concentration	Increase dilution of the sample or load less protein
	Exposed Too Long	Decrease exposure time
	Insufficient Washing	Ensure all the wash steps are carried out and the wash buffer is removed completely after each wash step
	Non-specific binding	Ensure the blocking buffer is stored and used properly.

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